

AMENDMENTS TO THE SPECIFICATION

Please amend the paragraph beginning on page 1, line 22 as follows:

It has been proved that the pathogen of the cold-water disease is ~~*Flavobacterium psychrophilium*~~ *Flavobacterium psychrophilum*, which is also known as ~~*Flexibacter cyelephils*~~ or ~~*Cytophagar cyelephils*~~ *Flexibacter psychrophilus* or *Cytophaga psychrophilia*. However, no vaccines against this disease have yet been developed.

Please amend the paragraph beginning on page 2, line 6 as follows:

The inventors of the present invention have investigated ~~*Flavobacterium psychrophilium*~~ *Flavobacterium psychrophilum* as a pathogen of the cold-water disease in terms of pathogenicity and vaccine activity depending on various cultivation conditions, and found a quite unexpectedly that the vaccine activity becomes higher by using bacteria in a logarithmic growth phase rather than by using bacteria in a stationary-state phase. The present invention has been completed based on this findings.

Please amend the paragraph beginning on page 2, line 15 as follows:

In a first aspect, the present invention provides the vaccine for cold-water disease in fish comprising inactivated cells of ~~*Flavobacterium psychrophilium*~~ *Flavobacterium psychrophilum* in a logarithmic growth phase or components of the cells.

Please amend the paragraph beginning on page 2, line 19 as follows:

In a second aspect, the present invention provides the vaccine composition for cold-water disease in fish containing inactivated cells of ~~*Flavobacterium psychrophilium*~~ *Flavobacterium psychrophilum* in a logarithmic growth phase or components of the cells.

Please amend the paragraph beginning on page 2, line 24 as follows:

In a third aspect, the present invention provides the method for preventing cold-water disease in fish comprising administering an effective dosage of inactivated cells of ~~*Flavobacterium psychrophilum*~~ *Flavobacterium psychrophilum* in a logarithmic growth phase or components of the cells.

Please amend the paragraph beginning on page 4, line 18 as follows:

Fig. 12 shows photographs of healthy rainbow trout in the control group (A), symptoms of dead rainbow trout that died on day 1 after challenge by immersion (B, C and D), symptoms of dead rainbow trout that died on day 5 after challenge by immersion (E and F), and ~~*Flavobacterium psychrophilum*~~ *Flavobacterium psychrophilum* found in the caudal fins of dead rainbow trout.

Please amend the paragraph beginning on page 5, line 2 as follows:

Inactivated cells of ~~*Flavobacterium psychrophilum*~~ *Flavobacterium psychrophilum* (may be referred to as the bacteria of the present invention hereinafter) in a logarithmic growth phase or components of the cells are used in the vaccine of the present invention. Usually, bacterial cultivation phases can be divided into a lag phase, logarithmic growth phase, stationary phase, extinction phase and survival phase. Many projections were observed on the surface of invading bacterial cells upon observation of the bacterial cells of the present invention invading fish bodies. On the other hand, differences of cell secretory products were detected by SDS-PAGE and the existence of projections was observed on the surface of the bacterial cells in the logarithmic growth phase upon observation of the

configuration and analysis of the bacteria of the present invention in the lag phase, logarithmic growth phase and stationary phase.

Please amend the paragraph beginning on page 6, line 2 as follows:

The carbon and nitrogen sources are not particularly restricted. Examples of them include tripton, serum of various animals, corn gluten meal, soy bean powder, corn steep liquor, casamino acid, yeast extract, pharma media, sardine meal, meat extract, peptone, ~~HiPro®, AjiPower®,~~ HIPRO (a gluten), AJIPOWER (an amino acid), corn meal, soy bean meal, coffee refuse, cotton seed oil refuse, ~~Cultivator®, Amiflex® and Ajipron®, Zest® and Ajix®~~ CULTIVATOR (a hydrolyzed vegetable protein), AMIFLEX (a hydrolyzed vegetable protein) and AJIPRON (a soy protein), ZEST (a vegetable ingredient) and AJIX (a vegetable protein). Examples of the carbon source include assimilable carbon sources such as arabinose, xylose, glucose, mannose, sucrose, maltose, soluble starch, lactose and cane molasses, and assimilable organic acids such as acetic acid. Phosphates, organic salts such as  $Mg^{2+}$ ,  $Ca^{2+}$ ,  $Mn^{2+}$ ,  $Zn^{2+}$ ,  $Co^{2+}$ ,  $Na^{+}$  and  $K^{+}$  salts, and other inorganic salts and trace amounts of nutrients, if necessary, may also be added to the culture medium. Commercially available culture media such as TY culture medium and Cytophagar (CYT) culture medium, as well as modified Cytophaga (MCYT) culture medium and culture medium supplemented with bovine fetal serum may also be used.

Please amend the paragraph beginning on page 9, line 2 as follows:

(1) Cells of ~~*Flavobacterium psychrophilum*~~ *Flavobacterium psychrophilum* G3724 (this strain was used in the experiments hereinafter) contained in a platinum loop were inoculated on a 4-mL MCYT culture medium (trypton 2.0 g, yeast extract 0.5 g, meat extract

0.2 g, sodium acetate 0.2 g, calcium chloride 0.2 g, distilled water 1000 mL, pH 7.2). After cultivation at 15°C for 2 days, a 0.5-mL fraction of the culture medium was inoculated on a 200-mL MCYT culture medium followed by cultivation with shaking at 15°C. The relationship between the cultivation time, and the cell number and optical density at 600 nm is shown in Fig. 1. Fig. 1 shows that the lag phase is from 0 to 24 hours after inoculating, the logarithmic growth phase is 24 to 48 hours after inoculating, and the stationary phase is after 48 hours from inoculating in the bacteria of the present invention.

Please amend the paragraph beginning on page 11, line 7 as follows:

~~*Flavobacterium psychrophilum*~~ *Flavobacterium psychrophilum* G3724 was cultured in 1000 mL of the MCYT culture medium contained in a 2000-mL Sakaguchi flask at 15°C. The cells showing OD 0.2 to 0.7 at 600 nm were used as the bacterial cells in the logarithmic growth phase. Then, the cells as a culture product at a growth phase showing OD of 0.2 to 0.7 at 600 nm in the culture period of 24 to 36-hour were inactivated by incubation in 0.3% formalin at 15°C for 2 days, and the inactivated bacterial cells were isolated by centrifugation at 4°C and 8,000 to 10,000 × g. The bacterial cells in the stationary phase after 36-hour cultivation (OD<sub>600nm</sub> = 1.0) were also inactivated by the same method as described above to obtain inactivated bacterial cells as controls.

Please amend the paragraph beginning on page 11, line 21 as follows:

Cells of ~~*Flavobacterium psychrophilum*~~ *Flavobacterium psychrophilum* G3724 contained in a platinum loop was inoculated on 50 mL of the MCYT culture medium and pre-cultured at 15°C for 48 hours. A 2.5-mL fraction of this culture medium was inoculated on 1000 mL of the MCYT culture medium, followed by culture at 15°C for 36 hours. OD at 600

nm was 0.2 to 0.7. The culture product was incubated in 0.3% formalin at 15°C for 2 days. The bacterial cells were then collected by centrifugation at 8,000 to 10,000 × g at 4°C. The cells obtained were re-suspended in physiological saline containing 0.3% formalin to obtain a vaccine suspension containing the inactivated bacterial cells of the present invention.

Please amend the paragraph beginning on page 15, line 22 as follows:

~~*Flavobacterium psychrophilum*~~ *Flavobacterium psychrophilum* NCMB 1947 was cultured with shaking in MCYT culture medium at 15°C, and the culture medium in the logarithmic growth phase was used for artificial infection when OD600 during 24 to 48 hours' cultivation reached 0.2 to 0.7. The bacteria of the present invention in the logarithmic growth phase were added to an aquarium of rainbow trout so that the concentration of the bacteria was  $10^6$  to  $10^8$  CFU/ml to attempt artificial infection by the immersion method. The body weight of rainbow trout used for the experiment was in the range of 1 to 4 g, and the water temperature was 15°C. As shown in Fig. 11, the mortality rate of the fish in the group infected with the bacteria of the invention in the logarithmic growth phase was 55.8%, in contrast to 0% in the control group (non-infection group). This result is the first successful artificial infection of rainbow trout by the immersion method. The photographs in Fig. 12 show healthy rainbow trout, symptoms of rainbow trout that died on day 1 (B, C and D) and on day 5 (E and F), and ~~*Flavobacterium psychrophilum*~~ *Flavobacterium psychrophilum* found in the caudal fins of dead rainbow trout (G and H).

Please delete the original Abstract appearing on page 18 and insert therefor the enclosed substitute Abstract as new page 18.